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## A tumor necrosis factor binding protein is present in human biological fluids

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Tumor necrosis factor (TNF) possesses both beneficial and toxic bioactivities. Mechanisms may operate to counteract harmful effects. We have identified a TNF binding protein (TNF-BP), which shows increased levels in serum and urine of patients on regular hemodialysis treatment (RDT). TNF-BP inhibited the specific binding of human recombinant TNF (rTNF) to its cell surface receptor. Results from gel chromatography demonstrated the presence in serum and urine of a macromolecule with an apparent molecular weight of 50000, which formed a complex with rTNF. A 62-fold purification of TNF-BP from urine of patients on RDT was achieved by ion exchange chromatography and gel chromatography. Partially purified TNF-BP reduced the growth inhibitory effect of rTNF on a susceptible leukemia cell line. TNF-BP may act as a regulator of the biological activity of TNF and could have beneficial effects in certain inflammatory conditions.

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### CLINICAL BEARING

Tumor necrosis factor (TNF) is produced by macrophages and was first identified because of its anticancer activity. Later it was seen to exert many effects in inflammation and immunity. Recent work has also implied that TNF may cause negative effects associated with inflammation, such as cachexia, tissue injury and shock. The finding of these potential harmful actions has led to an interest in agents which can block the actions of TNF. In this paper we described a TNF binding protein (TNF-BP), which was partially purified from urine of patients on regular hemodialysis. An agent like TNF-BP, when produced by recombinant DNA techniques, may become an important treatment for patients with septic shock and other harmful manifestations where TNF is involved.

Tumor necrosis factor (TNF), initially found in serum of mice and rabbits injected with bacillus Calmette-Guérin and endotoxin, was recognised because of its cytotoxic and antitumor properties (1). Numerous cell types which are targets for TNF have high-affinity surface receptors for this polypeptide (2). These receptors also combine with lymphotoxin (LT) (2-4). TNF may participate in the regulation of growth, differentiation and function of cells involved in inflammation, immunity and hematopoiesis (2, 5). TNF could therefore have beneficial effects for the host but it is also associated with harmful manifestations such as shock and tissue injury, which can be produced by administration of recombinant TNF

(rTNF) (6) and reversed by antibodies (7-8). The potentially harmful effects of TNF might be counteracted by as yet unidentified systems. A TNF-binding protein present in serum and urine, which we describe in the present communication, might be part of such a balancing system.

## Material and methods

**Tumor necrosis factor and lymphotoxin.** Recombinant human TNF (rTNF) and lymphotoxin (rLT) (produced by Genentech, Inc., San Francisco, CA) were supplied by Boehringer Ingelheim, Vienna, Austria. The rTNF contained  $38 \times 10^6$  U/mg (646 U/pmol) and the rLT  $230 \times 10^6$  U/mg (3960 U/pmol). For iodination of TNF, the two-phase method of Tejedor & Ballesta was used (9).

**Serum and urine samples from patients.** Blood was drawn from healthy individuals and patients on regular hemodialysis (RDT). Serum was collected after coagulation at room temperature for 2 h and stored at  $-20^\circ\text{C}$  until analyzed. Urine was dialyzed against 0.15 mol/l NaCl, 5 mmol/l 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), pH 7.4 with 1 g/l benzamidine-hydrochloride and stored at  $-20^\circ\text{C}$ .

**Assays for TNF-BP.** The presence of a TNF-BP was assayed both by competition with the  $^{125}\text{I}$ -rTNF binding to cells and by detection of complex formation by gel chromatography.

A subclone of HL-60 cells (HL-60-10) (5) was utilized for the competition assay. Cells ( $7.5 \times 10^6$ ) which had been maintained in suspension culture in RPMI-1640 medium with 10% fetal bovine serum (FBS) were washed with binding buffer (RPMI-1640, 10% FBS, 20 mmol/l HEPES, pH 7.4) and incubated with 50 pmol/l  $^{125}\text{I}$ -rTNF for 2 h at  $4^\circ\text{C}$  by rotation in 1.5 ml Eppendorf tubes in a total volume of 300  $\mu\text{l}$ . After centrifugation for 10 s at  $8000 \times g$  the pellet was resuspended and washed twice in ice-cold binding buffer to separate free and membrane-bound  $^{125}\text{I}$ -rTNF. The radioactivity of the pellet was measured in a  $\gamma$ -counter. Specific binding was defined as the difference between total binding and the non-specific binding that occurred in the presence of a 1000-fold excess of unlabelled rTNF. A standard curve was constructed from the inhibition caused by different volumes of dialyzed RDT urine by plotting the urine volume versus the ratio (bound  $^{125}\text{I}$ -rTNF)/(maximally bound  $^{125}\text{I}$ -rTNF) (B/B max). One unit (U) of TNF-BP was

defined as the amount of TNF-BP which reduced the ratio B/B max to 0.5.

In order to measure the effect of anti-TNF and anti-LT in the competition assay, antisera produced by immunization of rabbits were used. The immunoglobulin of 1  $\mu\text{l}$  of anti-TNF was adsorbed to 25 mg protein A-Sepharose (Pharmacia, Uppsala, Sweden) in 200  $\mu\text{l}$  of binding buffer at  $37^\circ\text{C}$  for 1 h. In order to remove TNF, which might be present in various biological samples, these were mixed with the anti-TNF-protein A-Sepharose in a final volume of 750  $\mu\text{l}$  and rotated at  $4^\circ\text{C}$  for 12 h. After centrifugation, the supernatant was used in the competition assay. This procedure completely eliminated the competition caused by 10 nmol/l rTNF. Anti-LT was found not to bind  $^{125}\text{I}$ -rTNF and therefore its effect could be determined directly by addition of 10  $\mu\text{l}$  of anti-LT to the biological sample plus incubation for 1 h at  $37^\circ\text{C}$  followed by competition assay. This procedure completely eliminated the inhibition of binding caused by 100 nmol/l rLT.

The gel chromatography assay was performed with serum and dialyzed urine samples (500  $\mu\text{l}$ ) incubated for 10 min at  $23^\circ\text{C}$  with  $^{125}\text{I}$ -rTNF at a final concentration of 200 pmol/l followed by chromatography on a Sephacryl 200 superfine column ( $1.5 \times 60$  cm) at  $4^\circ\text{C}$ . The column was eluted with 0.15 mol/l NaCl, 5 mmol/l HEPES, pH 7.4 at a flow rate of 25 ml/h. Mixtures were also incubated for another 10 min with a 250-fold excess of non-labelled rTNF (50 nmol/l) before gel chromatography.

**Partial purification of TNF-BP.** Urine from 1 patient, 300 ml, was concentrated by pressure ultrafiltration using Diaflo cells equipped with UM-10 membranes (Amicon Inc.), followed by dialysis against 10 mmol/l Tris HCl, pH 8.0 with 0.3% sodium azide.

**Step 1: Diethylaminoethanol (DEAE) - Sephacel chromatography.** The urine sample was applied on a DEAE-Sephacel column ( $1.5 \times 60$  cm). Elution was performed with a 150 ml NaCl/10 mmol/l Tris HCl, pH 8.0 gradient, ranging from 0 to 0.4 mol/l NaCl. The fractions containing TNF-BP as determined by the competition assay were pooled, lyophilized, resuspended and dialyzed against 0.15 mol/l NaCl, 5 mmol/l HEPES, pH 7.4.

**Step 2: Sephadex G-75 chromatography.** The TNF-BP of step 1 was chromatographed on a Sephadex G-75 superfine column ( $2.6 \times 90$  cm) equilibrated with 0.15 mol/l NaCl, 5 mmol/l HEPES, pH 7.4. The column

was eluted at a flow rate of 7 ml/h. Fractions containing TNF-BP as determined by the competition assay were pooled, lyophilized, resuspended and dialyzed against 0.15 mol/l NaCl, 5 mmol/l HEPES, pH 7.4 and filter sterilized. Protein was measured using the BioRad protein microassay procedure with albumin as a standard.

The pH stability of TNF-BP was investigated by incubation for 10 min in 0.15 mol/l NaCl, 5 mmol/l HEPES at pH 1.5 and pH 10.5 achieved by the addition of concentrated HCl and NaOH respectively. This procedure was followed by dialysis against 0.15 mol/l NaCl, 5 mmol/l HEPES, pH 7.4. Susceptibility to proteolytic digestion was determined by rotation of TNF-BP with 2.5 U/ml of insoluble Protease (from *Sterptomyces griseus*) at 37°C for 12 h followed by centrifugation and testing of the supernatant in the competition assay. A control sample was rotated without Protease. Susceptibility to periodate oxidation was determined by incubation of TNF-BP with 1 mmol/l sodium metaperiodate in the dark at room temperature for 10 min. The reaction was terminated by the addition of 10 mmol/l glycerol followed by extensive dialysis against 0.15 mol/l NaCl, 5 mmol/l HEPES pH 7.4.

**Assay for clonal growth inhibition by rTNF in agar culture.**  $2 \times 10^5$  HL-60-10 cells were seeded in 1 ml of 0.3% agar on top of 1 ml 0.5% agar in growth medium with 10% FBS in 35-mm tissue culture dishes and colonies were counted after 10 d.

**Statistical analysis.** Data were analyzed by Student's t-test if two measurements were compared and by a  $\chi^2$ -test if more than two measurements were compared.

## Results

**Demonstration of a TNF-BP in human serum and urine.** In experiments concerning the binding of  $^{125}$ I-rTNF to cells it was observed that serum and urine from patients on RDT contained a substance which reduced the binding. This substance is called TNF-binding protein (TNF-BP) because, as will be shown below, it binds to rTNF and is susceptible to digestion with protease.

Dialyzed urine from a patient on RDT gave a dose-dependent inhibition of the TNF-binding to HL-60-10 cells (Figure 1). One possible explana-

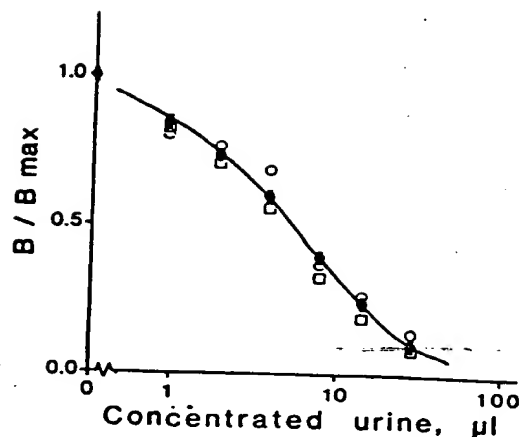


Figure 1. Inhibition of the binding of  $^{125}$ I-rTNF to HL-60-10 cells by dialyzed urine from patients on RDT. The binding (B) of 50 pmol/l  $^{125}$ I-rTNF was measured at 4°C in the presence of 20-fold concentrated and dialyzed urine from a patient on RDT (●—●). Bars indicate SEM (n = 6). One unit (U) of TNF-BP is defined as the amount which reduces the ratio B/B max to 0.5. The inhibition observed was not due to the presence in the urine of TNF itself or of LT because anti-TNF antibodies (○) or anti-LT antibodies (□) did not influence the inhibition (n = 2).

tion for the inhibition would be the presence in the urine of TNF itself or of LT, which competes with the labelled TNF for binding (2, 4). These possibilities were ruled out by the finding that it was not possible to remove the inhibition of binding by adsorption of the urine sample to anti-TNF antibodies bound to protein A-Sepharose or by the addition of neutralizing antibodies to LT (Figure 1). No inhibition of binding was observed when the cells were preincubated with the dialyzed RDT urine for 20 min at 37°C and washed twice in ice-cold binding buffer before addition of  $^{125}$ I-rTNF (data not shown). This result demonstrates that the inhibitor does not act by binding to the cells. TNF-BP was not detectable in normal urine by this assay. Pooled serum from healthy individuals contained approximately 1 U/ml, whereas urine from RDT patients (n = 20) contained 3–27 U/ml and serum from RDT patients (n = 42) contained 7–40 U/ml of TNF-BP.

The formation of a complex between rTNF

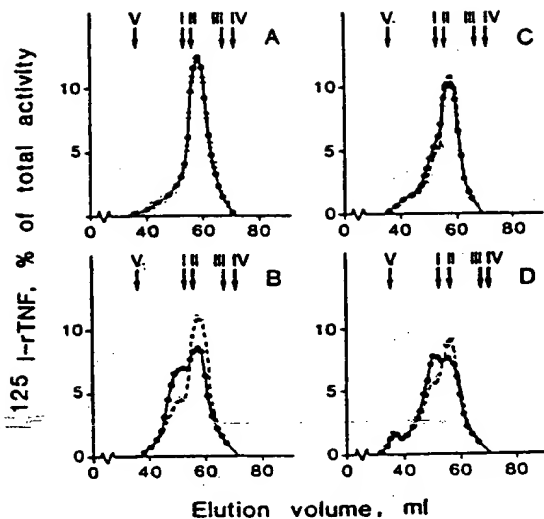


Figure 2. Complex formation between  $^{125}\text{I}$ -rTNF and a TNF-BP in human serum and urine. A: Gel chromatography on Sephacryl 200 of 200 pmol/l  $^{125}\text{I}$ -rTNF in elution buffer ( $\square$ — $\square$ ) and of 200 pmol/l  $^{125}\text{I}$ -rTNF in dialyzed urine from a healthy individual ( $\bullet$ — $\bullet$ ). B: Elution profiles of 200 pmol/l  $^{125}\text{I}$ -rTNF in dialyzed urine from a patient on RDT in the absence ( $\bullet$ — $\bullet$ ) and presence ( $\circ$ — $\circ$ ) of a 250-fold excess of unlabelled rTNF. C: Elution profiles of 200 pmol/l  $^{125}\text{I}$ -rTNF in serum from a healthy individual in the absence ( $\bullet$ — $\bullet$ ) and presence ( $\circ$ — $\circ$ ) of a 250-fold excess of unlabelled rTNF. D: Elution profiles of mixtures of 200 pmol/l  $^{125}\text{I}$ -rTNF with serum from a patient on RDT in the absence ( $\bullet$ — $\bullet$ ) and presence ( $\circ$ — $\circ$ ) of a 250-fold excess of unlabelled rTNF. Arrows indicate void volume ( $V_0$ ), and elution volumes of bovine serum albumin,  $M_r 67000$  (I), ovalbumin,  $M_r 43000$  (II), chymotrypsinogen,  $M_r 25000$  (III) and ribonuclease,  $M_r 13700$  (IV).

and a substance in biological fluids was demonstrated by gel chromatography. Free  $^{125}\text{I}$ -rTNF eluted as a single peak corresponding to a molecular weight of approximately 35000 (Figure 2A). No complex formation was seen with urine

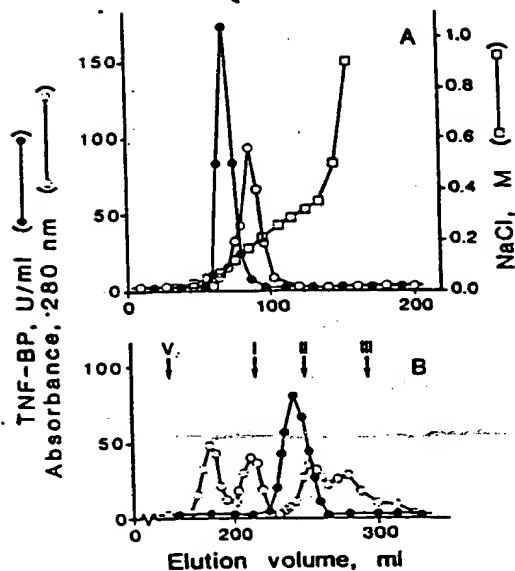


Figure 3. Partial purification of TNF-BP from urine. Dialyzed RDT urine (300 ml) was fractionated by ion exchange chromatography on a DEAE-Sephacel column ( $1.5 \times 60$  cm) eluted with a NaCl gradient ( $\square$ — $\square$ ) (A). Fractions containing TNF-BP were pooled and concentrated and chromatographed on a Sephadex G-75 superfine column ( $2.6 \times 90$  cm) (B). TNF-BP; ( $\bullet$ — $\bullet$ ). Absorbance at 280 nm ( $\circ$ — $\circ$ ), (in A  $\times 0.3$ , in B  $\times 0.03$ ). Molecular weight markers are indicated as in Figure 2.

from healthy individuals as all  $^{125}\text{I}$ -rTNF eluted as free TNF (Figure 2A). In urine from a patient on RDT (Figure 2B), serum from a healthy individual (Figure 2C) or serum from a patient on RDT (Figure 2D),  $^{125}\text{I}$ -rTNF eluted in two major peaks, one with a molecular weight of approximately 75000, and another with a molecular weight of approximately 35000. A substance is present in these biological fluids which binds to rTNF, resulting in a complex with a molecular weight of approximately 75000. Thus normal

TABLE I  
Partial purification of TNF-BP from urine of a patient on RDT

	TNF-BP (U)	Protein (mg)	Yield	Specific activity (U/mg protein)	Purification
Dialyzed urine	2900	645	1.0	4.5	1
DEAE-Sephacel	1870	86	0.6	22	4.9
Sephadex G-75	1530	5.5	0.5	280	62

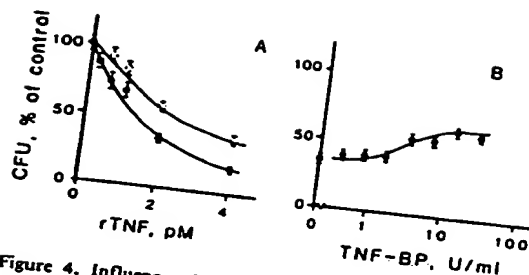


Figure 4. Influence of TNF-BP on the growth inhibition of rTNF. A: Inhibition by rTNF on the clonal growth of HL-60-10 cells in the absence (●—●) and presence (○—○) of 13 U/ml TNF-BP. B: Dose-dependent effect of TNF-BP on the growth inhibitory effect of a constant concentration of 2 pmol/l rTNF. Bars indicate SEM ( $n = 6$ ).

serum but not normal urine contains detectable levels of TNF-BP. Complex formation was partially inhibited by a 250-fold excess of non-labelled rTNF (Figure 2). This result shows that both labelled and nonlabelled rTNF bind to TNF-BP and makes it unlikely that the fraction with a molecular weight of 75000 represents an oligomer form of rTNF.

**Partial purification and physicochemical characterization of TNF-BP.** The results of ion exchange and gel chromatography are shown in Figure 3. TNF-BP was eluted with approximately 0.1 mol/l NaCl from the DEAE-column and an apparent molecular weight of 50000 was calculated from the results of gel chromatography. A 62-fold purification was achieved by these chromatography steps with a yield of 0.5 (Table 1).

The partially purified TNF-BP was used for physicochemical studies. TNF-BP was resistant to incubation for 10 min at pH 1.5 and pH 10.5 since 91% and 87% respectively of the activity was recovered. TNF-BP was, however, susceptible to treatment with protease from *Streptomyces griseus*, which destroyed 82% of its biological activity ( $p < 0.001$ ). After periodate oxidation of TNF-BP, 76% of its bioactivity remained.

**Inhibition of the biological activity of rTNF by TNF-BP.** TNF-BP (13 U/ml) reduced ( $p < 0.001$ ) the growth inhibition of rTNF on HL-60-10 cells

in agar culture (Figure 4). TNF-BP alone did not interfere with cell growth. Similar results were obtained when the TNF-BP concentration was varied between 0.4 and 27 U/ml with a constant concentration of 2 pmol/l rTNF (Figure 4B).

## Discussion

The presence in human serum and RDT urine of a TNF-binding protein was demonstrated by several experiments. Human serum and dialyzed urine from patients on RDT inhibited the specific binding of  $^{125}$ I-rTNF to cells. The inhibition observed did not result from the presence of TNF or LT in the biological fluids since neutralizing antibodies to rTNF and rLT did not influence the inhibitory capacity of the fluids. Data from gel chromatography showed the presence in the biological samples of a macromolecule with an apparent molecular weight of 50000, which formed a stable complex with rTNF. Finally, partially purified TNF-BP inhibited the biological activity of rTNF. The reversal of TNF biological activity on clonal growth was not complete. The final effect was most likely affected by the long incubation time of 10 d. Short-term assays will be necessary to obtain detailed information on the biological effects of TNF-BP.

TNF-BP of biological fluids could be present in two forms, free and in complex with TNF. It is important to realize that our assays detect only free TNF-BP. Therefore it is possible that we underestimate the content of TNF-BP in serum and urine.

As human TNF is a nonglycosylated protein (10), rTNF and native TNF are probably identical, indicating that TNF-BP would bind native TNF as well. The bioactivity of TNF-BP was resistant to low or high pH but susceptible to protease treatment. The binding to TNF was probably independent of oligosaccharide side chains on TNF-BP as it was unaltered after treatment of TNF-BP with periodate.

TNF-BP in serum and urine from patients on RDT are most likely identical, since the molecular size of the complex with rTNF was independent of the source of TNF-BP, and increased levels were

found both in serum and urine from the patients. Proteins of the size of TNF-BP normally pass the glomerular filter in the kidney, and are largely taken up and catabolized by tubular cells (11). In nephron loss disease, both excretion and endogenous catabolism are diminished, leading to accumulation of these proteins in blood as well as in urine (11).

Recently Seckinger et al (12) reported the presence of a TNF inhibitory activity in urine from some febrile patients. This activity was demonstrated by use of a cytotoxicity assay with a TNF-susceptible cell line in the presence of actinomycin D. It was sensitive to heat and trypsin, with an apparent molecular weight of 40000–60000. The TNF-BP of the present work and the TNF inhibitory activity reported by Seckinger et al (12) are therefore most probably identical.

An inhibitor for interleukin 1 (IL-1) has been found in febrile urine (13–15). This inhibitor, which does not interfere with the action of TNF (4), competes with IL-1 for binding to its cell surface receptor (16). Uromodulin can bind to both rIL-1 and rTNF (17). The higher molecular weight of uromodulin (85000) and its absence in serum indicate that it is not identical to TNF-BP.

TNF-BP may act as a regulator of the biological activity of TNF as it seems to have the capacity of buffering changes in free TNF. Consequently, addition of TNF-BP to human biological fluids will down-regulate their TNF activity which might have some beneficial effects in certain inflammatory conditions. It will be important to consider the possibility that TNF-BP may represent a soluble form of the TNF receptor.

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